

RESEARCH

Mutation of the first ATG ORF-3 of Chicken Anemia Virus into ACG completely abolish the apoptin production

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Abstract

To test the effect of one point mutation on the first initiation codon of Chicken Anemia Virus (CAV) open reading frame-3 (ORF-3), an apoptin knocked out expressor plasmid pCLS-VP3(-) and a CAV apoptin knocked out plasmid pCAV/Ap(-) were constructed. In both plasmids, the first ATG in ORF-3 was mutated into ACG by a reversed long PCR. No apoptin detected in COS-1 cells transfected with pCLS-VP3(-) using western blotting and immunofluorescence assay, while apoptin was detected in COS-1 cells transfected with pCLS wild type. After released from pCAV/Ap(-), the complete genome of CAV/Ap(-) was ligated to form the replicative form. The apoptin production was completely abolished in MDCC-MSB1 cells transfected with replicative form of CAV/Ap(-). The apoptin production was fully regained after a reverse mutation into CAV/Ap(-)RM. These data shows the first evidence that mutation of the first ATG of ORF-3 into ACG could completely abolish the production of apoptin.

Key words: Chicken Anemia Virus, Circovirus, Gyrovirus, Apoptin

INTRODUCTION

Chicken Anemia Virus (CAV) was first isolated in Japan ⁽¹⁾. CAV causes anemia in newborn chicken by apoptosis of hemocytoblasts in the bone marrow ⁽²⁾. CAV also induces apoptosis of cortical thymocytes and lymphoblastoid cell lines ⁽³⁾. CAV is a small non-enveloped virus with a single-stranded circular DNA genome in the size of approximately 2.3 kb ^(4, 5). Currently, CAV is the only member of genus *Gyrovirus* within family *Circoviridae*. However, while circoviruses have ambisense genomes, CAV has an antisense genome. CAV also lacks the 9-nt stem-loop structure common in Circovirus ^(4, 6, 7).

The configurations of ORFs in CAV were similar with torque teno virus, a human virus found in serum from a hepatitis patient with the initials T.T ⁽⁸⁾. These two viruses have a more than 80 % similarity in a 36-nt stretch

near the replication origin, but nucleotide sequences of the remaining genome show no apparent similarities ^(7, 9). Their transcription patterns to produce three or more spliced mRNAs are also similar to each other ⁽¹⁰⁻¹²⁾.

CAV replicates via a circular double-stranded replicative form (RF) ⁽⁴⁾. The major transcript of CAV is an unspliced polycistronic mRNA which possesses three overlapping ORFs, encoding VP1 (52kDa), VP2 (24kDa), and VP3 (14 kDa) ^(4, 13). The capsid of CAV contains only the VP1 ⁽¹⁴⁾. VP-2 has been shown to interact with VP1 and has dual phosphatase activities ⁽¹⁵⁾. VP-3, known as apoptin, induces apoptosis selectively in transformed cells but not in non-transformed

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cells ^(16, 17). In point of its apoptosis activity, reports make progress. However, the function of apoptin in CAV in its replication has not been elucidated yet.

One strategy to study the role of apoptin in CAV replication is by a mutagenesis study, i.e. by knocking out the apoptin by introducing one point mutation in its initiation codon. However, the open reading frame for apoptin is overlap with the open reading frame for VP2. Furthermore, no evidence for apoptin production after mutation in its first ATG has been ever given to the best of my knowledge. The present study provided the first evidence of the mutation of first ATG of CAV ORF-3 into ACG could abolish the production of apoptin.

MATERIALS AND METHODS

Cell Culture

Chicken T cell transformed by Marek's Disease Virus, MDCC-MSB1, were used to replicate CAV. Cells were subcultured twice weekly at 39 °C in RPMI1640 medium (Sigma, Stenheim, Germany) supplemented with 10 % fetal bovine serum (FBS, Immuno-Biological Laboratories, Fujioka, Japan), 2 mM glutamine, and 0.01 % each of penicillin and streptomycin. African green monkey kidney cell expressing SV40T antigen, COS-1, were used to visualize localization of apoptin and its mutant. Cells were subcultured twice weekly at 37 °C in Dulbecco's modified Eagle medium supplemented with 10 % FBS, 2 mM glutamine and 0.01 % each of penicillin and streptomycin.

Wild-type CAV Plasmid and Apoptin Knocked Out Mutants

A molecular clone of the wild-type CAV (CAV/WT), pCAV-A2C15 (pCAV/WT) (Genbank AB031296), was kindly supplied by Dr. Yamaguchi, National Institute of Animal Health, Japan ⁽¹⁸⁾. pCAV/Ap(-), a mutant plasmid contains a CAV clone knocked out of apoptin, was constructed as described elsewhere ⁽¹⁹⁾. Briefly, a point mutation, A⁴⁶⁵TG to ACG, was introduced to the initiation codon of apoptin gene on the frame 3. The mutation was designed to be synonymous in VP2 on the

frame 2. A reverse mutant, pCAV/ApRM, was prepared using pCAV/Ap(-) as a template, as described elsewhere ⁽¹⁹⁾.

pCLS-VP3(-)

Another plasmid to confirm the mutation result was constructed as described elsewhere (Prasetyo, A.A., et al, submitted for publication). Briefly, the chicken anemia virus leader sequence was cloned and inserted into pHM6 vector (Roche Diagnostic, Mannheim, Germany). The clone thus obtained, pCLS, driven by CMV promotor could expressed a wild-type apoptin in animal cells, i.e. COS-1 cells. The initiation codon of ORF-3 was introduced using same strategy as above using pCLS as template, resulted pCLS-VP3(-), a apoptin knocked out plasmid.

Preparation of Replicative Form (RF) DNA

Each molecular clone of CAV in an amount of 5.0 µg was digested with XbaI. After electrophoresis on a 1 % agarose, viral DNA was extracted by the gel extraction kit (Qiagen, Hilden, Germany), and the DNA was circularized by the T4 DNA ligase. DNA was extracted with phenol-chloroform (1:1) and precipitated with ethanol. The pellet was washed with 70 % ethanol, dried, and resolved in filtered TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Aliquot was tested in agarose to confirm consistent ligation.

DNA Transfection

MDCC-MSB1 cells (1 x 10⁶ cells/2 ml) in RPMI medium free from antibiotics were delivered to the 35 mm dish. The RF DNA (200 ng) was transfected with FuGENE HD (Roche) according to manufacturer's protocol. COS-1 cells (2 x 10⁵ cells/2 ml) in DMEM medium free from antibiotics were delivered to the 35 mm dish 12 h prior of transfection. The pCLS and pCLS-VP3(-) (1 µg each) were transfected with FuGENE HD. All culture was harvested at 72 h posttransfection.

Western Blot Analysis

Transfected cells were lysed in 200 µL of running buffer (0.125 M Tris-HCl pH 6.8, 10 % 2-mercaptoethanol, 4 % SDS, 10 % sucrose,

0.004 % bromophenol blue) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred from the gel to Immobilon-P (Millipore) with a dry blotting system. Transfer was performed in 192 mM glycine- 25 mM Tris -20 % ethanol for 60 min at 60 mA/cm². Filters were blocked, washed, incubated with antibodies and developed with ECL Western Blotting Detection Reagents in accordance with the suppliers's protocol (Amersham Biosciences). A mouse anti-apoptin monoclonal antibody (TropBio, Australia) was used to detect apoptin expression.

Immunofluorescence

The cells (one night prior to transfection for COS-1 cells or at the day of transfection for MDCC-MSB1-cells) were delivered into 8-well chamber slides. Cells were transfected with 1 µg of plamid with FuGENE HD, air-dried at 48 hours posttransfection, and fixed with 4 % paraformaldehyde in PBS(-) for 10 min at room temperature. After consecutive washings with PBS(-) and 0.1 % Triton X-100 in PBS(-), the slides were blocked with 15 µL/well of 1.5 % normal sheep serum (in PBS(-)) at 37 °C for 30 min. The cells were incubated with 1/100 dilution of anti-VP3 monoclonal antibody (TropBio, Queensland, Australia), and then with 1/1000 horse radish peroxidase anti-mouse IgG antibody (Amersham), each for 60 minutes at room temperature. The slides were mounted with 5 ng (5 µL)/well of Vectorshield with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, CA), and observed under a inverted fluorescent microscope (BX60: Olympus, Tokyo, Japan). The specimen was thoroughly washed with PBS(-) between each step unless otherwise stated. The apoptin sublocalization was analyzed using ImageJ, a public domain analysis software.

RESULTS AND DISCUSSION

The initiation codon for apoptin located +133 nucleotides downstream of the trascription start point (nt 333), or 107 nucleotides downstream of the VP2 initiation codon. The stop codon for apoptin is located at

nt 827 while ORF-2 is at nt-1007, therefore the apoptin's ORF is overlapping with ORF for VP2. Although the initiation codon for apoptin is in not high favourable sequence context ⁽²⁰⁾, the apoptin could expressed efficiently in MDCC-MSB1 infected with CAV, in MDCC-MSB1 cells transfected with replicative form of CAV, or in cells transfected with an apoptin expression plasmid ^(16, 21, 22).

To knock out the apoptin, a point mutation was introduced into its initiation codon. The ATG was mutated into ACG in order to avoid any amino acid change in the overlapping VP2. Two different systems were used for this experiment, by using the replicative form of CAV and the expression plasmid.

First, the effect of mutation in apoptin initiation codon was tested in COS-1 cells. The CAV leader sequence from nt 333 until 831 was subcloned into a vector, pHM6. The human cytomegalovirus immediate early promoter / enhancer in this vector plasmid could ensure the efficien and high-level expression of recombinant protein in mammalian cell lines (e.g. COS-1 cells). The plasmid thus obtained, pCLS, contains CAV leader sequence with apoptin ORF on it, downstream of the CMV promoter. After transfected with pCLS, a band in apoptin size was detected using Western blot (Figure I-A). This result consistent with previous result, that apoptin could be expressed from an apoptin expression plamid ⁽²¹⁾. The sublocalization of apoptin also was checked. In COS-1 cells, > 60% of cells expressing apoptin showed a nuclear dominant staining (data not shown), consistent with previous reports ⁽²³⁻²⁶⁾.



Figure 1. Mutation of the first initiation codon of apoptin could abolished apoptin production. A = pCLS ; B = pCLS-VP3(-) ; C = Replicative form CAV/WT ; D = Replicative form CAV/Ap(-) reverse mutant

The effect of one point mutation in apoptin initiation codon was first tested in COS-1 cells, using pCLS-VP3(-). As expected,

no band in apoptin size was detected using Western blot (Figure I-B), and no cells expressing apoptin was found in immunofluorescence detection (data not shown). This result indicated that mutation of the apoptin initiation codon from ATG into ACG could abolish the expression of apoptin.

Next, replicative form of CAV was used to confirm the result. One may argue that what happened in COS-1 cells using apoptin expression plasmid may not represent the result in CAV replication origin. After MDCC-MSB1 cells transfected with replicative form of CAV wild type (CAV/WT), a band in apoptin size was detected using Western blot (Figure I-C), and cells expressing apoptin was also detected using immunofluorescence assay. Small band detected in Western blot data may due of protein breakdown caused by the highly positively charged N-terminal region of apoptin. However, apoptin intracellular distributions could not be visualized using immunofluorescence assay because of scanty cytoplasm in floating MDCC-MSB1 cells (data not shown). Using pCAV/WT, an apoptin knocked out mutant, named as pCAV/Ap(-) was constructed. After released from the backbone plasmid, the CAV/Ap(-) genom was circularized and transfected into MDCC-MSB1 cells. As expected, there was no apoptin detected using western blotting nor immunofluorescence assay (data not shown). To confirm the result, a reverse mutant, CAV/Ap(-)RM was constructed and its replicative form was transfected into MDCC-MSB1 cells. As expected, apoptin could fully regained in this reverse mutant (Figure I-D). Taken together, these data indicated that one point mutation on apoptin initiation codon, from ATG into ACG, could completely abolish the expression of apoptin.

Recently, apoptin was found inevitable for CAV replication ⁽¹⁹⁾. Apoptin is important not only for DNA replication but also for virus particle formation. No infective virus detected in MDCC-MSB1 cells transfected with replicative form of CAV/Ap(-) nor the signal from its DNA replication. In addition, a reverse mutant with the reorganized apoptin gene obtained from CAV/Ap(-), CAV/ApRM, regained full recovery in the infectious virus production. The data presented in this study

strongly support the recent result about inevitability of apoptin for CAV replication.

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